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Toxicovenomics and antivenom profiling of the Eastern green mamba
snake (*Dendroaspis angusticeps*)

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32 **Abstract**

33 A toxicovenomic study was performed on the venom of the green mamba,
34 *Dendroaspis angusticeps*. Forty-two different proteins were identified in the venom of
35 *D. angusticeps*, in addition to the nucleoside adenosine. The most abundant proteins
36 belong to the three-finger toxin (3FTx) (69.2%) and the Kunitz-type proteinase inhibitor
37 (16.3%) families. Several sub-subfamilies of the 3FTxs were identified, such as Orphan
38 Group XI (Toxin F-VIII), acetylcholinesterase inhibitors (fasciculins), and aminergic
39 toxins (muscarinic toxins, synergistic-like toxins, and adrenergic toxins). Remarkably,
40 no α -neurotoxins were identified. Proteins of the Kunitz-type proteinase inhibitor
41 family include dendrotoxins. Toxicological screening revealed a lack of lethal activity
42 in all RP-HPLC fractions, except one, at the doses tested. Thus, the overall toxicity
43 depends on the synergistic action of various types of proteins, such as dendrotoxins,
44 fasciculins, and probably other synergistically-acting toxins. Polyspecific antivenoms
45 manufactured in South Africa and India were effective in the neutralization of venom-
46 induced lethality. These antivenoms also showed a pattern of broad immunorecognition
47 of the different HPLC fractions by ELISA and immunoprecipitated the crude venom by
48 gel immunodiffusion. The synergistic mechanism of toxicity constitutes a challenge for
49 the development of effective recombinant antibodies, as it requires the identification of
50 the most relevant synergistic toxins.

51 (197 words)

52 **Keywords:** *Dendroaspis angusticeps*; Green mamba; Snake venom; Proteomics;
53 Toxicovenomics; Antivenoms.

54

55 **Biological significance**

56 Envenomings by elapid snakes of the genus *Dendroaspis*, collectively known as
57 mambas, represent a serious medical problem in sub-Saharan Africa. The development
58 of novel antivenoms and of recombinant neutralizing antibodies demands the
59 identification of the most relevant toxins in these venoms. In this study, a bottom-up
60 approach was followed for the study of the proteome of the venom of the Eastern green
61 mamba, *D. angusticeps*. Forty-two different proteins were identified, among which the
62 three-finger toxin (3FTx) family, characteristic of elapid venoms, was the most
63 abundant, followed by the Kunitz-type proteinase inhibitor family. In addition, several
64 other protein families were present in the venom, together with the nucleoside
65 adenosine. No α -neurotoxins were identified within the family of 3FTxs in the venom
66 of *D. angusticeps*, in contrast to the venom of *D. polylepis*, in which α -neurotoxins are
67 largely responsible for the toxicity. With one exception, HPLC fractions from *D.*
68 *angusticeps* venom did not kill mice at the doses tested. This underscores that the
69 toxicity of the whole venom is due to the synergistic action of various components, such
70 as fasciculins and dendrotoxins, and probably other synergistically-acting toxins. Thus,
71 the venoms of these closely related species (*D. angusticeps* and *D. polylepis*) seem to
72 have different mechanisms to subdue their prey, which may be related to different prey
73 preferences, as *D. angusticeps* is predominantly arboreal, whereas *D. polylepis* lives
74 mostly in open bush country and feeds mainly on mammals. It is therefore likely that
75 the predominant clinical manifestations of human envenomings by these species also
76 differ, although in both cases neurotoxic manifestations predominate. Polyspecific
77 antivenoms manufactured in South Africa and India were effective in the neutralization
78 of venom-induced lethality in mice and showed a pattern of broad immunorecognition
79 of the various venom fractions. It is necessary to identify the toxins responsible for the

80 synergistic mode of toxicity in this venom, since they are the targets for the
81 development of recombinant antibodies for the treatment of envenomings.

82

83 **1. Introduction**

84 The Eastern green mamba (*Dendroaspis angusticeps*) is a highly venomous
85 elapid found primarily in southeastern Africa (Figure 1). First described by Smith in
86 1848 [1], *D. angusticeps* is a relatively small mamba species, averaging 1.4 m in length.
87 Due to its arboreal, shy, and elusive nature, human envenomings are less frequent than
88 those inflicted by the more territorial *Dendroaspis polylepis* (black mamba) [2,3]. Adult
89 specimens of *D. angusticeps* have a brilliant emerald to lime green coloration, providing
90 them with an excellent camouflage in their natural habitat of the tropical rainforests in
91 the coastal lowlands of Southeast Africa [4]. *D. angusticeps* is, however, also found in
92 areas with coastal bush, dune, and montane forest [5], as well as in closer proximity to
93 humans, when residing in farm trees, such as citrus, mango, coconut, and cashew [6].
94 Due to its color and habitat, *D. angusticeps* is often mistaken for a harmless tree snake,
95 why people often do not take proper precaution [2,3]. *D. angusticeps* preferably preys
96 on warm-blooded animals, such as rodents, bats, birds, and nestlings, but also on eggs
97 [4].

98 Despite a low number of human envenomings reported, but due to its potent
99 neurotoxic venom, *D. angusticeps* is classified as a category 1 snake, which is the
100 highest level of medically important snakes, according to the WHO [7]. Furthermore, its
101 high abundance, particularly in Kenya, Tanzania, Mozambique, Malawi, eastern
102 Zimbabwe, and the Republic of South Africa, makes this a snake of high
103 epidemiological relevance [7]. Severe envenomings by *D. angusticeps* can lead to rapid
104 mortality within only 30 minutes of a bite [6]. The typical clinical manifestations
105 include swelling of the bitten area, dizziness, nausea, difficult breathing, irregular
106 heartbeat, and respiratory paralysis [6]. These life-threatening symptoms may escalate
107 rapidly, but deaths are rare when effective antivenom is administered timely [6].

108 Given the medical importance of *D. angusticeps*, it is necessary to have a
109 thorough understanding of the composition of its venom, as well as of the underlying
110 mechanisms for venom pathophysiology in human victims. Furthermore, preclinical
111 assessment of antivenoms is critical for predicting efficacy of snakebite envenoming
112 therapy, which may be used to guide clinicians in the treatment of snakebites by *D.*
113 *angusticeps*. Currently, only the SAIMR Polyvalent Snake Antivenom from the South
114 African Vaccine Producers is claimed to be effective against *D. angusticeps*, although it
115 is possible that other polyvalent antivenoms raised against the venoms of other mamba
116 species may be effective in neutralization of *D. angusticeps* venom.

117 The venom of *D. angusticeps* has not undergone a full proteomics evaluation,
118 and its quantitative protein composition is not known. Nevertheless, several biochemical
119 and pharmacological studies have been performed on different toxins from *D.*
120 *angusticeps* venom [8–11]. These studies report that this venom contains several
121 neurotoxins, such as the fasciculins [10] and dendrotoxins [8,9], which are unique to the
122 *Dendroaspis* genus [12,13]. This venom also contains a number of other toxins of the
123 three-finger toxin family (3FTx), such as muscarinic toxins, adrenergic toxins, and
124 synergistic-type toxins [14–16].

125 The dendrotoxins, of structural similarity to the Kunitz-type serine protease
126 inhibitors, target the presynaptic voltage-gated potassium channels with high specificity,
127 facilitating the release of acetylcholine from the presynaptic nerve terminals, causing
128 excitatory activity [17,18]. Other important neurotoxins of the 3FTx family present in
129 *D. angusticeps* venom are the fasciculins, which prolong the presence of acetylcholine
130 in the neuromuscular junction by inhibiting acetylcholinesterase, leading to muscle
131 fasciculations [10]. Although the venom composition of *D. angusticeps* has not been
132 elucidated, a study of the venom of the closely related and more feared relative, *D.*

133 *polylepis* (black mamba), has recently been reported [19]. According to this study, *D.*
134 *polylepis* venom is dominated by α -neurotoxins from the 3FTx family and dendrotoxins
135 (BPTI-type/Kunitz type protease inhibitors). It is therefore of relevance to study the
136 venom proteome of *D. angusticeps* in order to identify similarities and differences with
137 that of *D. polylepis*.

138 Toxicovenomics defines the recent convergence between toxicological
139 evaluation of toxins and venomics [20,21]. Together with antivenomics, this tool may
140 help provide a better understanding of *D. angusticeps* venom, the relative importance of
141 different proteins for toxicity, and how venom toxicity may best be abrogated. While
142 previous investigations of *D. angusticeps* have focused on the biochemical and
143 pharmacological features of the toxins, recent advances in the field of venomics and
144 antivenomics facilitate development of novel antivenoms through rational and
145 knowledge-based interpretation of pharmacological relevant toxins [22].

146 Here, we report the first full toxicovenomics analysis of *D. angusticeps*, a
147 quantitative estimation of its venome, and a preclinical and immunochemical
148 assessment of three antivenoms against *D. angusticeps* venom.

149

150 **2. Materials and Methods**

151 *2.1 Snake venom*

152 Venom from *D. angusticeps* was obtained from Latoxan SAS, Valence, France,
153 from a pool of 50 specimens collected in Tanzania.

154

155 *2.2 Venom separation by reverse-phase HPLC and SDS-PAGE*

156 The ‘snake venomics’ analytical strategy [23] involving fractionation of crude
157 venom by a combination of RP-HPLC and SDS-PAGE separation steps, was followed.

Venom (2 mg) was dissolved in 200 μ L of water containing 0.1% trifluoroacetic acid (TFA; solution A) and separated by RP-HPLC (Agilent 1200) on a C₁₈ column (250 x 4.6 mm, 5 μ m particle; Teknokroma). Elution was carried out at 1 mL/min by applying a gradient towards solution B (acetonitrile, containing 0.1% TFA): 0% B for 5 min, 0–15% B over 10 min, 15–45% B over 60 min, 45–70% B over 10 min, and 70% B over 9 min, as previously described [24]. Fractions, collected manually, were dried in a vacuum centrifuge, redissolved in water, reduced with 5% β -mercaptoethanol at 100 °C for 5 min, and further separated by SDS-PAGE in 15% gels. Proteins were stained with colloidal Coomassie blue G-250, and gel images were acquired on a ChemiDoc[®] recorder using ImageLab[®] software (Bio-Rad).

2.3 Protein identification by tandem mass spectrometry of tryptic peptides

Protein bands were excised from the polyacrylamide gels and subjected to reduction (10 mM dithiothreitol), alkylation (50 mM iodoacetamide), and overnight in-gel digestion with sequencing grade trypsin (Sigma), in 50 mM ammonium bicarbonate at 37 °C. Tryptic peptides were extracted with 50% acetonitrile containing 1% TFA, and analyzed by MALDI-TOF-TOF on an AB4800-Plus Proteomics Analyzer (Applied Biosystems). Peptides were mixed with an equal volume of saturated α -cyano-hydroxycinnamic acid (in 50% acetonitrile, 0.1% TFA), and spotted (1 μ L) onto an Opti-TOF 384-well plate, dried, and analyzed in positive reflector mode. TOF spectra were acquired using 500 shots at a laser intensity of 3000. Selection of the ten most intense precursor ions was done automatically and their TOF/TOF fragmentation spectra were acquired using 500 shots at a laser intensity of 3900. External calibration in each run was performed with CalMix[®] standards (ABSciex) spotted onto the same plate. Resulting spectra were searched against the UniProt/SwissProt database for

Serpentes (20150217) using ProteinPilot[®] v.4 and the Paragon[®] algorithm (ABSciex) at $\geq 95\%$ confidence, or, in few cases, manually interpreted and the deduced sequences searched using BLAST (<http://blast.ncbi.nlm.nih.gov>) for protein family assignment by similarity.

2.4 Relative protein abundance estimations

Relative abundance of the venom proteins was estimated by integrating the areas of their chromatographic peaks at 215 nm, roughly corresponding to peptide bond abundance, using the ChemStation[®] software (Agilent) [23]. In the case where HPLC peaks contained several electrophoretic bands, their percentage distributions were assigned by densitometry, using ImageLab[®] (Bio-Rad). Finally, for electrophoretic bands in which more than one protein was identified by MALDI-TOF-TOF, their percentage distributions were estimated on the basis of the corresponding intensities of the intact protein ions, as observed in the nESI-MS analysis. Intensities lower than 5% (relative to the major protein ions in such mixtures) were considered as traces.

2.5 Adenosine analysis

The presence of the nucleoside adenosine was determined by spiking a sample of 2 mg of venom with 10 μ g of adenosine and separating it by RP-HPLC as described in section 2.2. If the adenosine coincided with a peak already present in a crude venom sample (as judged by the increment in the height of the peak), and if this venom peak showed an ESI-MS spectrum essentially identical to adenosine, the identity of venom component was judged to be adenosine. Further confirmation of the molecular identity of adenosine was obtained by acquiring its collision-induced dissociation MS/MS spectrum in positive mode, using the Enhanced Product Ion tool of Analyst v1.5 in the

QTrap3200 mass spectrometer, to show the expected reporter ion transition 268 → 136. Nucleoside abundance was estimated by deriving un-spiked nucleoside concentration from integrating the areas of both spiked and un-spiked chromatographic peaks.

2.6 *In vitro* enzymatic activities

2.6.1. *Phospholipase A₂* activity

Assay of PLA₂ activity was carried out using the monodisperse synthetic chromogenic substrate 4-nitro-3-octanoyloxybenzoic acid (NOBA) [25]. Twenty-five µL of solution containing various amounts of venom were mixed with 200 µL of 10 mM Tris, 10 mM CaCl₂, 0.1 M NaCl, pH 8.0, and 25 µL of NOBA to achieve a final substrate concentration of 0.32 mM. Plates were incubated at 37 °C for 60 min, and absorbances were recorded at 405 nm in a microplate reader. For comparative purposes, the activities of the venoms of *D. polylepis* and the viperid snake *Bothrops asper* were also assessed.

2.6.2 *Proteinase* activity

Proteinase activity was assayed by adding 20 µg of venom to 100 µL of azocasein (10 mg/mL in 50 mM Tris–HCl, 0.15 M NaCl, 5 mM CaCl₂ buffer, pH 8.0), and incubated for 90 min at 37 °C. The reaction was terminated by addition of 200 µL of 5% trichloroacetic acid, and after centrifugation (5 min, 6000 g), 150 µL of supernatants were mixed with 100 µL of 0.5 M NaOH, and absorbances were recorded at 450 nm. The absorbance of azocasein incubated with distilled water alone was used as a blank, being subtracted from all readings [26]. For comparative purposes, the activities of the venoms of *D. polylepis* and the viperid snake *Bothrops asper* were also assessed.

233

234 2.7 Toxicological profiling

235 2.7.1 Animals

236 *In vivo* assays were performed in CD-1 mice of both sexes, provided by Instituto
237 Clodomiro Picado, following protocols approved by the Institutional Committee for the
238 Use and Care of Animals (CICUA), University of Costa Rica. Mice were provided food
239 and water *ad libitum*.

240

241 2.7.2 Toxicity of crude venom and isolated venom fractions

242 The lethality of the whole venom and venom fractions was tested by intravenous
243 (i.v.) injection in groups of four mice (18–20 g body weight). Various amounts of
244 venom or venom fractions were dissolved in phosphate-buffered saline (PBS; 0.12 M
245 NaCl, 0.04 M sodium phosphate buffer, pH 7.2) and injected in the caudal vein, in a
246 volume of 100 μ L. Deaths occurring within 24 h were recorded, and LD₅₀s were
247 calculated by probits [27], using the BioStat[®] software (AnalySoft).

248 The acute toxicity of venom fractions was initially screened by selecting a dose
249 based on fraction abundance in the venom and assuming a venom yield of 75 mg for *D.*
250 *angusticeps* (<http://snakedatabase.org/pages/LD50.php#legendAndDefinitions>),
251 Laustsen et al.'s Toxicity Score [20], and 50 kg as the weight of a human being. On this
252 basis, a cutoff dose (mg/kg) was selected and tested for each fraction. Fractions that
253 were not lethal at this dose (corresponding to a Toxicity Score below 7) were considered
254 as having insignificant acute toxicity, whereas fractions which did kill mice at this level
255 were further evaluated, and precise LD₅₀s were determined for them.

256

257 2.8 Antivenoms

Polyspecific antivenoms from the following manufacturers were used: (a) SAIMR (South African Institute for Medical Research) Polyvalent Snake Antivenom from South African Vaccine Producers (Pty) Ltd (batch number BC02645, expiry date 07/2016); (b) Snake Venom Antivenom (Central Africa) from VINS Bioproducts Ltd (batch 12AS13002, expiry date 04/2017); (c) Snake Venom Antivenom (African) from VINS Bioproducts Ltd (batch 13022, expiry date 01/2018). In addition, the monospecific *Micrurus nigrocinctus* Anticoral Antivenom from Instituto Clodomiro Picado (batch 5310713ACLQ, expiry date 07/2016) was used for comparison in certain experiments.

2.9 Immunoreactivity of antivenoms against crude venom and venom fractions by ELISA

Wells in MaxiSorp plates (NUNC, Roskilde, Denmark) were coated overnight with 1.0 µg of each HPLC venom fraction, or crude venom, dissolved in 100 µL PBS. After a washing step, wells were blocked by adding 100 µL PBS containing 2% (w:v) bovine serum albumin (BSA, Sigma), and incubated at room temperature for 1 h. Plates were then washed five times with PBS. A dilution of each antivenom in PBS + 2% BSA was prepared. 100 µL of antivenom solution was added to each well in triplicates and incubated for 2 h. Plates were then washed five times with PBS. 100 µL of a 1:2000 dilution of conjugated antibody (Sigma A6063, rabbit anti-horse IgG (whole molecule)-alkaline phosphatase in PBS + 1% BSA) was then added to each well. The plates were incubated for 2 h, and then washed five times with FALC buffer (0.05 M Tris, 0.15 M NaCl, 20 µM ZnCl₂, 1 mM MgCl₂, pH 7.4). Development of color was attained by addition of 100 µL *p*-nitrophenyl phosphate (1 mg/mL in 9.7% v/v diethanolamine buffer, pH 9.8) and absorbances at 405 nm were recorded (Multiskan FC, Thermo Scientific).

2.10 Double immunodiffusion of antivenoms against venoms from *D. polylepis* and *D. angusticeps*

Agarose was dissolved in 30 mL of PBS to attain 1% concentration, and poured into a Petri dish. Six holes were punched in the gel, and 50 µl of antivenom was placed in the center well, while 30 µl of solutions of *D. angusticeps* and *D. polylepis* venoms were added to the surrounding wells at variable concentrations (0.5, 1 and 2 µg/µL). After overnight incubation at room temperature, agarose gels were photographed using a ChemiDoc[®] recorder and ImageLab[®] software.

2.10 Neutralization studies with antivenoms

Mixtures containing a fixed amount of venom and variable dilutions of antivenoms were prepared using PBS as diluent and incubated at 37 °C for 30 min. Controls contained PBS instead of antivenom. Aliquots of 100 µL of the solutions, containing 4 LD₅₀s of venom (64 µg/mouse), were then injected i.v. into groups of four mice (18-20 g). Deaths occurring within 24 h were recorded for determining the neutralizing capacity of antivenoms. Neutralization was expressed as the Median Effective Dose (ED₅₀) of antivenom, defined as the ratio mg venom/mL antivenom at which 50% of the injected mice were protected. The ED₅₀s as estimated by probits, as described in Section 2.6.2.

3.0 Results and Discussion

3.1 Venomics

SDS-PAGE separation of venom proteins revealed similarities and differences between the venoms of *D. angusticeps* and *D. polylepis* (Figure 2). Both venoms

showed predominantly low molecular mass bands, in addition to a number of bands of a wide range of molecular masses, including some large proteins with molecular masses above 100 kDa. When SDS-PAGE was run under non-reducing conditions, the venom of *D. angusticeps* showed more bands than that of *D. polylepis*. In particular, *D. angusticeps* venom presented three bands of molecular masses between 18 and 22 kDa, which were absent in the venom of *D. polylepis*. In turn, *D. polylepis* venom had a band of 37 kDa, absent in *D. angusticeps* venom. In contrast, with the exception of a 25 kDa band in the venom of *D. angusticeps*, the majority of these intermediate molecular mass bands were not observed in reduced gels, indicating that these bands were comprised of higher order protein complexes.

A bottom-up proteomic characterization of *D. angusticeps* venom was carried out. Using RP-HPLC, the venom was resolved into 29 fractions, where the first three eluting from the column did not contain proteins as evidenced by electrophoresis. After SDS-PAGE separation, the remaining 26 fractions were resolved into 63 bands (Figure 3), of which 59 resulted in positive identifications upon in-gel digestion and MALDI-TOF-TOF analysis, whereas 4 remained unknown. In total, 42 different proteins were identified within these bands (Table 1). In certain cases, exemplified by fraction number 5, the bands separated by SDS-PAGE contained the same protein in both monomer and dimer forms.

Fractions 1-3 did not contain proteins according to SDS-PAGE. Due to its high abundance, fraction 1 was analyzed by direct infusion using nESI-MS/MS, which revealed a component with a molecular mass of 268 Da. Upon collision-induced dissociation, this ion produced a fragment of 136 Da, corresponding to the characteristic transition of adenosine. Furthermore, spiking with adenosine as described in section 2.5, provided an estimation that 0.75% of the chromatographic signal of the venom

333 corresponded to this nucleoside. Presence of a substantial amount of adenosine was also
334 observed in the venom of *D. polylepis* [19]. Adenosine might play an auxiliary role in
335 the toxicity of mamba venoms owing to its vasodilatory effect, as previously suggested
336 [19].

337 The overall protein composition of *D. angusticeps* venom was determined by
338 assigning the identified proteins to families and expressing these as percentages of total
339 protein content (Figure 4). The most abundant components belong to the three-finger
340 toxin family (3FTx; 69.2%) and the family of Kunitz-type proteinase inhibitors, which
341 includes the dendrotoxins (KUN; 16.3%). The 3FTxs in elapid venoms all share a
342 common structural architecture with a distinct protein fold, comprising between 60 and
343 80 amino acids in length, containing a small, globular, hydrophobic core with four or
344 five conserved disulfide bridges, from which three β -stranded loops extend [28–30].
345 This makes this group of toxins resemble three outstretched fingers [31]. Despite the
346 common structural motif, a diverse array of functions has been associated with 3FTxs
347 [32].

348 All 3FTxs found in *D. angusticeps* venom belong to the short chain subfamily,
349 but attained to different sub-subfamilies (Figure 4). The majority of 3FTxs in this
350 venom belong to the Orphan Group XI (from Toxin FV-III), whose function has not yet
351 been established [29], followed by aminergic toxins (Muscarinic toxin 2, Muscarinic
352 toxin 4, Synergistic-like protein, and Adrenergic toxins) [16]. A further 8.4% of 3FTxs
353 were attained to fasciculins (all from the acetylcholinesterase inhibitory sub-subfamily),
354 which are unique to *D. angusticeps* [33].

355 Interestingly, the proteomic analysis of the 3FTxs of *D. angusticeps* venom did
356 not reveal the presence of α -neurotoxins, perhaps the most studied 3FTxs from elapid
357 venoms. α -neurotoxins bind with high affinity to the nicotinic cholinergic receptor at

the motor end-plate of the neuromuscular junction, causing a blockage in neuromuscular transmission and flaccid paralysis, generally inducing death by respiratory failure [34]. α -neurotoxins show the highest Toxicity Score values among the fractions of *D. polylepis* venom [19]. Their absence in the venom of *D. angusticeps* marks a significant difference between these two mamba venoms and suggests that the predominant mechanisms for prey immobilization in these venoms might be different.

Another type of neurotoxins unique to the *Dendroaspis* genus, and found in our proteomic analysis of *D. angusticeps* venom, is comprised by the dendrotoxins, which are homologous to Kunitz-type serine proteinase inhibitors [35]. Dendrotoxins interact and inhibit the presynaptic voltage-gated potassium channels, thus exerting a facilitatory effect associated with excitability [18,35]. The venom of *D. angusticeps* has a lower relative content of Kunitz-type proteinase inhibitors, but a higher content of 3FTxs, when compared to the venom of *D. polylepis* [19]. The combined action of the fasciculins and dendrotoxins results in enhanced skeletal muscle excitability and contraction, probably leading to respiratory arrest.

Other protein families found in lower proportions in the venom of *D. angusticeps* include metalloproteinases (SVMP; 6.7%), cysteine-rich secretory proteins (CRISP; 2.1%), and traces of Galactose-binding lectins (GAL; < 0.5%), peptidases (PEP; < 0.1%), hyaluronidases (HYA; < 0.3%), and nerve growth factors (NGF < 0.1%) (Figure 4). An extremely low PLA₂ activity was observed *in vitro* for *D. angusticeps* venom (Figure 5A), in agreement with previous findings [36]. The proteomic analysis, however, did not identify any PLA₂ in this venom, implying that such enzyme would be present only in trace amounts. Alternatively, the very low PLA₂ activity recorded for this venom may correspond to low levels of non-specific hydrolysis of the NOBA synthetic substrate caused by other enzymes. The negligible

content of PLA₂s in *Dendroaspis* venoms contrasts with the characteristic high amounts and activity of this enzyme in many other elapid venoms [37,38]. Also, despite the presence of 6.7% of SVMPs in the venom proteome, very low proteinase activity was observed for *D. angusticeps* venom when using azocasein as substrate (Figure 5B). This observation mirrors the negligible activity described for *D. polylepis* venom [19]. It is likely that *Dendroaspis* SVMPs have a restricted substrate specificity, as occurs in SVMPs from other elapid venoms [39,40].

3.2. Toxicity of venom fractions

Toxicity testing was performed for most venom fractions (Table 2). Using the Toxicity Score defined by Laustsen et al. [20], a cut-off Toxicity Score value of 7, below which a fraction would be deemed to not be of medical relevance for lethality, was chosen for screening the fractions. From Table 2 it is evident that the vast majority of the fractions did not induce lethality in mice when tested individually. Only fraction 8 (containing Rho-elapitoxin-Da1b and Fasciculin-2) was shown to be lethal at the doses tested, with an LD₅₀ of 0.58 mg/kg (95% confidence limits: 0.17-1.23 mg/kg) and a toxicity score of 10.9. A previous study showed that an ‘angusticeps-type’ toxin, which corresponds to a fasciculin, induced respiratory arrest in mice within minutes after an i.v. injection of a dose of 1 mg/kg, and also caused cardiovascular alterations [41]. Nevertheless the Toxicity Score of fraction 8 contrasts with the overall Toxicity Score of 117.6 for the whole venom, suggesting that different toxins in *D. angusticeps* venom may act in a synergistic manner, thereby potentiating each other’s toxic effects, leading to higher toxicity for whole venom. To further investigate the possible synergism between toxins in the venom, fractions 4-12 were combined in equivalent amounts (according to mass), and the LD₅₀ was determined to be 1.36 mg/kg (95%

confidence limits: 0.96-1.66 mg/kg), corresponding to a Toxicity Score of 51.7, providing further evidence for the presence of synergism. The identity of the toxins acting synergistically is presently unknown; however, it is suggested that fasciculins and dendrotoxins, and probably other synergistically acting proteins, might be involved in this phenomenon. It should be kept in mind that the solvents used in RP-HPLC separation, particularly acetonitrile, denature some venom components, especially SVMPs; thus, the toxicity of SVMP fractions cannot be assessed with our approach. Nevertheless, elapid SVMPs are unlikely to play a key role in lethality. In support of this, it was previously shown that the LD₅₀ of *D. polylepis* venom was not significantly altered after incubating venom with RP-HPLC solvents [19].

Despite its lack of α -neurotoxins, the venom of *D. angusticeps* is quite effective in killing mice rapidly after injection, as observed in our toxicity experiments with crude venom, where the controls receiving 4 LD₅₀s of venom on average died within 10 minutes. Previous studies highlighted two main toxic activities when *D. angusticeps* whole venom is tested in experimental systems. On various nerve-muscle preparations, this venom augmented the responses to indirect stimulation [35], possibly due to the combined action of dendrotoxins and fasciculins. Then, prolonged exposure to higher venom concentrations resulted in failure of muscle contraction. Additionally, the venom induced hypotension in various animal models, an effect that was blocked by the muscarinic cholinergic antagonist atropine [42]. This effect could be caused by the 3FTxs, previously characterized from this venom, that act on muscarinic cholinergic and adrenergic receptors [14–16]. Thus, the combined action of the various neurotoxin types present in *D. angusticeps* venom may result in a complex series of neuromuscular and cardiovascular effects, which result in effective prey immobilization in the absence of the action of α -neurotoxins. This toxicological scenario, and the existence of

synergistic effects, complicates the selection of the most relevant toxins towards which antibodies should be raised in order to abrogate venom toxicity. This challenging task demands the identification of the most relevant synergistic toxins.

3.3 Immunoprofiling and neutralizing ability of antivenoms

Three polyspecific antivenoms, which are distributed in sub-Saharan Africa, were investigated for their ability to neutralize *D. angusticeps* venom and their ability to recognize both whole venoms and venom fractions. The SAVP antivenom showed the highest neutralizing ability against *D. angusticeps* venom, with an ED₅₀ (mg venom neutralized per mL antivenom) of 4.0 mg/mL (95% confidence limits: 1.7-10.0 mg/mL). VINS African antivenom also neutralized the lethal activity of the venom, with an ED₅₀ of 2.4 mg/mL (95% confidence limits: 1.4-4.0 mg/mL). On the other hand, VINS Central African antivenom failed to neutralize *D. angusticeps* venom at the lowest venom/antivenom ratio tested (1.0 mg venom/mL antivenom). These results bear a relationship with the fact that the venom of *D. angusticeps* is included in the immunization mixture for the manufacture of SAVP antivenom, whereas the two VINS antivenoms do not include this venom during immunization. The two VINS products do, however, include the venoms of other *Dendroaspis* species, according to their leaflet information. Gel immunodiffusion tests of the three antivenoms indeed revealed that cross-reactive antigens between *D. angusticeps* and *D. polylepis* venoms exist, evidenced by the SAVP antivenom, which produced the strongest precipitin bands with identity or partial identity patterns (Figure 6). Cross-reactivity between at least some components of these two venoms would explain the neutralization obtained with the VINS antivenom, despite these being produced without using *D. angusticeps* venom. It would be relevant to perform detailed studies on the antigenic relationships of the main

toxicologically-relevant components of *Dendroaspis* venoms, such as the various types of 3FTxs and dendrotoxins, in order to have a knowledge base for selecting the venoms or toxins to be used for preparing antivenoms. Interestingly, gel immunodiffusion results, regarding the intensity of precipitates, showed a better correlation with the neutralization potencies observed for the three antivenoms compared to their ELISA titration curves against immobilized crude venoms, which showed only minor differences in binding among them (Figure 7). Although the SAVP antivenom displays a slightly stronger binding when comparing the three antivenoms on the basis of volume, differences are less evident when the antivenoms are evaluated based on their protein concentrations (Figure 7). In general, solid-phase immunoassays of antivenoms against crude venoms do not always predict their neutralizing efficacy, as antibodies may bind to highly immunogenic venom components that may not have a key role in toxicity.

To further investigate the immunorecognition patterns of the antivenoms, binding of their antibodies to the different venom fractions was measured by ELISA. From Figure 8, it is evident that a somewhat similar recognition pattern exists for the different antivenoms. However, not only does the SAVP antivenom in general display stronger binding to the venom fractions compared to the VINS antivenoms, but SAVP antivenom also shows a much stronger binding to the fractions in the first part of the chromatogram (4-10), containing the 3FTxs and the dendrotoxins. These findings, based on the use of immobilized venom fractions rather than crude venoms, better agree with the *in vivo* neutralization studies described above.

Observations performed on mice injected with mixtures of venom and antivenom in the neutralization experiments revealed that, at some venom/antivenom ratios, mice were protected from death, but nevertheless showed evident manifestations

of toxicity, such as reduced mobility (without paralysis) and congestion of the eyes. This suggests that toxins responsible for these effects are not fully neutralized, at some of the tested venom/antivenom ratios. Since these toxins may play an important role in envenomings, it would be relevant to assess whether these non-lethal manifestations of toxicity are neutralized or not in the evaluation of an antivenom. For instance, in the case of SAVP antivenom, complete neutralization of lethality and of these additional manifestations was observed at a venom/antivenom ratio of 1.0 mg/mL. In contrast, at ratios of 2 and 3 mg venom/mL antivenom, lethality was abrogated, but reduced mobility and eye congestion were present to some extent. At ratios of 4 mg venom/mL antivenom and higher, lethality was not completely neutralized. Similar observations were performed with VINS African antivenom, whereby complete neutralization of lethality and the other effects was achieved at 0.5 mg venom/mL antivenom, whereas at 1 mg/mL the additional effects were observed, and lethality was abrogated. These findings underscore the relevance of identifying the most relevant toxins in the venom of *D. angusticeps* in order to ensure that neutralizing antibodies against them are included in heterologous or recombinant antivenoms in the future.

4.0 Concluding remarks and outlook

The venom proteome of *D. angusticeps* was characterized by a bottom-up approach. It shows a predominance of 3FTxs and Kunitz-type proteinase inhibitors, with additional less abundant components of various protein families. A remarkable feature of this venom is the absence of α -neurotoxins, in sharp contrast with the venom of the closely related species *D. polylepis*. The toxicity analysis of RP-HPLC fractions revealed that only one fraction was lethal to mice at the doses tested, and that the lethality of whole venom was much higher than what would be expected based on the

lethality of individual fractions. This highlights the presence of synergism between various venom components, such as dendrotoxins, fasciculins, and probably aminergic 3FTxs of various types. South African polyvalent antivenom and one Indian antivenom were effective in the neutralization of venom lethality, in agreement with a pattern of immunorecognition of the various RP-HPLC fractions. On the basis of the synergism observed in the overall toxicity of this venom, the development of an effective combination of recombinant neutralizing antibodies demands the identification of the most relevant synergistic toxins that need to be neutralized – a task that awaits future research efforts.

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Figure legends

Figure 1: (A) *Dendroaspis angusticeps* (B) Distribution of *D. angusticeps* in Africa.

Figure 2: SDS-PAGE comparing crude venom of *Dendroaspis angusticeps* and *D. polylepis* under non-reduced (A) and reduced (B) conditions. Various amounts of each venom were separated in 15% gels and stained with Coomassie Blue G-250. Molecular mass markers (M) are labeled to the right, in kDa.

Figure 3: Separation of *Dendroaspis angusticeps* venom proteins using RP-HPLC (A), followed by SDS-PAGE (B). Two mg of venom were fractionated on a C₁₈ column and eluted with an acetonitrile gradient (dashed line), as described in Methods. Further separation of protein fractions was performed by SDS-PAGE under reducing conditions. Molecular weight markers (M) are indicated in kDa. Coomassie-stained bands were excised, in-gel digested with trypsin, and subjected to MALDI-TOF/TOF analysis for assignment to protein families, as shown in Table 1.

Figure 4: Composition of the *Dendroaspis angusticeps* venom proteome according to protein families (A) and three-finger toxin sub-subfamilies (B), expressed as percentages of total protein content. **KUN**: Bovine pancreatic trypsin inhibitors/Kunitz inhibitors (dendrotoxins); **3FTx**: Three-finger toxins; **SVMP**: Metalloproteinases; **GAL**: Galactose-binding lectins; **PEP**: Peptidases; **HYA**: Hyaluronidases; **KTC**: Prokineticins; **NGF**: Nerve growth factors. **CRISP**: Cysteine-rich secretory proteins. *Proteins in this fraction (Mambalgins) are not classified to a sub-subfamily; however they are known to inhibit acid sensing ion channels. **MIX**: Fractions of different

members of the 3FTx family for which percentages were not determined; subfamilies in this group include: Aminergic toxin, Antiplatelet toxin, Orphan group XI, and Acid sensing ion channel inhibitor.

Figure 5: (A) Comparison of the phospholipase A₂ activity of 20 µg of the venoms of *Dendroaspis angusticeps*, *Dendroaspis polylepis*, and *Bothrops asper*, on 4-nitro-3-octanoyloxybenzoic acid synthetic substrate. (B) Comparison of the proteolytic activity of 40 µg of venoms of *D. angusticeps*, *D. polylepis*, and *B. asper*, on azocasein substrate. Venoms from both species of *Dendroaspis* show extremely low phospholipase A₂ and proteinase activities. Each bar represents mean ± SD of triplicates.

Figure 6: Gel immunodiffusion assay of antivenoms against the venoms of *Dendroaspis angusticeps* (Da) and *Dendroaspis polylepis* (Dp). Antivenoms (50 µL) were added to the central wells, and solutions of various concentrations of venoms (30 µL) were added to peripheral wells. (A): VINS African antivenom. (B): VINS Central Africa Antivenom. (C): SAVP antivenom. (D): *Micrurus nigrocinctus* antivenom.

Figure 7: ELISA titrations of antivenoms against immobilized crude venoms of *Dendroaspis angusticeps* (A and C) and *Dendroaspis polylepis* (B and D) **SAVP:** SAIMR Polyvalent Snake Antivenom from South African Vaccine Producers, **VINS African:** Snake Venom Antiserum (African) from VINS Bioproducts Ltd., **VINS Central Africa** Snake Venom Antiserum (Central Africa) from VINS Bioproducts Ltd. Normal horse serum was used as a negative control. Each point represents mean ± SD

697 of triplicate wells. Antivenom titrations are represented as volumetric dilutions in A and
698 B, or as protein concentrations in C and D.

699

700 **Figure 8:** ELISA-based immunoprofiling of antivenoms against HPLC fractions of
701 *Dendroaspis angusticeps* venom. Binding of the equine antibodies to the immobilized
702 venom fractions was detected as described in Methods. Normal horse serum was used as
703 a negative control. For identification of venom fractions see Table 2. **(A) SAVP:**
704 SAIMR Polyvalent Snake Antivenom from South African Vaccine Producers. **(B):**
705 **VINS African:** Snake Venom Antiserum (African) from VINS Bioproducts Ltd., **VINS**
706 **Central Africa** Snake Venom Antiserum (Central Africa) from VINS Bioproducts Ltd.
707 Each bar represents mean \pm SD of triplicate wells.

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7b.i	1.1	14.0	1557.7	1	ALLTNGENSCYR	99	10	3FTx	<i>D. angusticeps</i> ; P81946
7b.ii			1253.6	1	MIWTYDGVIR	99	10	3FTx	Dendrotoxin A (fragm) <i>D. angusticeps</i> ; Q9PS08
7c.i	4.6	12.0	2821.2 1253.6 1409.7	1 1 1	LICYNQLGTKPPTTETCGDDSCYK MIWTYDGVIR MIWTYDGVIR	99 99 98	12 16 10	3FTx	Thrombostatin <i>D. angusticeps</i> ; P18328
7c.ii	trace		1356.6	1	FDWSGCGGNSNR	99	11	BPTI/KUN	Thrombostatin <i>D. angusticeps</i> ; P81946
8a	3.0	13.7	1517.6 1304.5 1344.6 1685.8 1557.7	1 1 1 1 1	GCGCPPGDDNLEVK CCTSPDKCNY TMCYSHITTSR ALLTNGENSCYRK ALLTNGENSCYR	99 99 99 99 99	7 10 10 15 17	3FTx	Alpha-dendrotoxin <i>D. angusticeps</i> ; P00980
8b.i	3.4	11.5	2426.2	1	DTHFGITQNCPPAGQNLCEIR	99	13	3FTx	Fasciculin-2 <i>D. angusticeps</i> ; P0C1Z0
8b.ii			1253.6	1	MIWTYDGVIR	97.7	6	3FTx	Rho-elapioxin-Dalb <i>D. angusticeps</i> ; P86419
9a	1.8	15.8	1507.7	1	SIGGISTEECAAGQK	99	8	3FTx	Thrombostatin <i>D. angusticeps</i> ; P81946
9b	3.2	12.9	1557.7 1685.8 1344.6	1 1 1	ALLTNGENSCYR ALLTNGENSCYRK TMCYSHITTSR	99 93.6 50.2	12 6 4	3FTx	Synergistic-like protein <i>D. angusticeps</i> ; P17696
9c.i	4.4	11.0	1507.7	1	SIGGISTEECAAGQK	99	6	3FTx	Fasciculin-1 <i>D. angusticeps</i> ; P0C1Y9
9c.ii	6.6		1216.4	1	CL(E ^{dh})FTYGGCK	99	11	BPTI/KUN	Synergistic-like protein <i>D. angusticeps</i> ; P17696
10a	0.6	15.7	1557.8	1	ALLTNGENSCYR	68.8	5	3FTx	Protease inhibitor 1 <i>W. Aegyptia</i> ; C11C50
10b	1.6	12.9	1304.5 1344.6 1566.7	1 1 1	CCTSPDKCNY TMCYSHITTSR GCGCPPGDDYLEVK	99 99 99	9 12 18	3FTx	Dendrotoxin A (fragm) <i>D. angusticeps</i> ; Q9PS08
									Fasciculin-1 <i>D. angusticeps</i> ; P0C1Y9

10c.i	4.6	10.8	1685.8 1557.7 1165.7 1507.8	1 1 1 1	ALLTNGGENSCYRK ALLTNGGENSCYR MGPKLYDVSR SIGGISTEECAQOK	99 99 99 99	14 16 7 13	3FTx 3FTx	Synergistic-like protein <i>D. angusticeps</i> ; P17696
10c.ii			1253.7	1	MIWITYDGVIR	99	7	3FTx	Thrombostatin <i>D. angusticeps</i> ; P81946
11a.i	1.3	15.1	1288.7 2035.0	1 1	EMLVAIHCCR GCGCPSKEMLVAIHCCR	99 99	10 11	3FTx	Toxin F-VIII <i>D. angusticeps</i> ; P01404
11a.ii	trace		1356.6	1	FDWSSGCGGNSNR	96.7	6	BPTI/KUN	Alpha-dendrotoxin <i>D. angusticeps</i> ; P00980
11b.i	6.5	11.5	1288.7	1	EMLVAIHCCR	99	11	3FTx	Toxin F-VIII <i>D. angusticeps</i> ; P01404
11b.ii	trace		2724.4	1	VCTPVGTSGEDCHPASHKIPIFSGQR	99	10	KTC	Toxin MIT1 <i>D. polylepis</i> ; P25687
11b.iii	trace		1557.8	1	ALLTNGGENSCYR	99	6	3FTx	Dendrotoxin A (fragm) <i>D. angusticeps</i> ; Q9PS08
11c.i	9.0	10.4	1288.7 1281.6 2035.0	1 1 1	EMLVAIHCCR EMLVAIHCCR GCGCPSKEMLVAIHCCR	99 99 99	14 12 15	3FTx	Toxin F-VIII <i>D. angusticeps</i> ; P01404
11c.ii	trace		2434.1	1	GCGCPTAMWPYQTECCCKGDR	99	17	3FTx	Toxin S4C8 <i>D. jamesoni</i> ; P25683
12a	1.2	15.3	1645.9	1	WQPPWYCKEPIVR	80.2	5	BPTI/KUN	Kunitz-type calcicludeine <i>D. angusticeps</i> ; P81658
12b.i	trace	11.1	1294.7 2724.4 2209.1 2296.1	1 1 1 1	GTCCAVSLWIK VCTPVGTSGEDCHPASHKIPIFSGQR MHHTCPCAPNLACVQTSPIK VCTPVGTSGEDCHPASHKIPIF	99 99 99 91.8	12 13 26 6	KTC	Toxin MIT1 <i>D. polylepis</i> ; P25687
12b.ii	3.0		1441.7	1	FCYHNIGMPFR	99	11	3FTx	Mambalgin-3 <i>D. angusticeps</i> ; C0HJB0
12c.i	trace	10.1	1645.9 1669.8	1 1	WQPPWYCKEPIVR CLPFLFSGCGGNANR	99 99	11 15	BPTI/KUN	Kunitz-type calcicludeine <i>D. angusticeps</i> ; P81658

				1797.9	1	KCLPFLFSGGCGGNANR	98.7	7		
12c.ii	3.5			2034.9	1	GGCPSKEMLV/AlHCCR	99	13	3FTx	Toxin F-VIII <i>D. angusticeps</i> ; P01404
				1288.7	1	EMLV/AlHCCR	99	14		
13a.i	trace	24.4		1669.8	1	CLPFLFSGGCGGNANR	99	7	BPTI/KUN	Kunitz-type calcicludeine <i>D. angusticeps</i> ; P81658
13a.ii	0.4****			1288.6	1	EMLV/AlHCCR	99	11	3FTx	Toxin F-VIII <i>D. angusticeps</i> ; P01404
13a.iii				1441.7	1	FCYHNIGMPFR	99	13	3FTx	Mambalgin-3 <i>D. angusticeps</i> ; C0HJB0
13b.i	trace	19.8		1669.8	1	CLPFLFSGGCGGNANR	99	10	BPTI/KUN	Kunitz-type calcicludeine <i>D. angusticeps</i> ; P81658
				1645.8	1	WQPPWYCKEPPVR	99	9		
13b.ii	1.1****			1288.6	1	EMLV/AlHCCR	99	9	3FTx	Toxin F-VIII <i>D. angusticeps</i> ; P01404
				2034.9	1	GGCPSKEMLV/AlHCCR	99	19		
13b.iii				1441.7	1	FCYHNIGMPFR	99	15	3FTx	Mambalgin-3 <i>D. angusticeps</i> ; C0HJB0
13c.i	trace	15.4		1669.8	1	CLPFLFSGGCGGNANR	99	13	BPTI/KUN	Kunitz-type calcicludeine <i>D. angusticeps</i> ; P81658
				1645.8	1	WQPPWYCKEPPVR	99	12		
13c.ii	2.9****			2034.9	1	GGCPSKEMLV/AlHCCR	99	16	3FTx	Toxin F-VIII <i>D. angusticeps</i> ; P01404
				1288.6	1	EMLV/AlHCCR	99	9		
13c.iii				1441.7	1	FCYHNIGMPFR	99	16	3FTx	Mambalgin-3 <i>D. angusticeps</i> ; C0HJB0
14a	0.4	19.1				Negative				
14b.i	0.1	15.4		1797.9	1	KCLPFLFSGGCGGNANR	99	16	BPTI/KUN	Kunitz-type calcicludeine <i>D. angusticeps</i> ; P81658
				1669.8	1	CLPFLFSGGCGGNANR	99	17		
				1645.8	1	WQPPWYCKEPPVR	99	13		
				1164.5	1	WQPPWYCK	95.1	5		
14b.ii	1.2			2034.9	1	GGCPSKEMLV/AlHCCR	99	13	3FTx	Toxin F-VIII <i>D. angusticeps</i> ; P01404
14b.iii				1288.6	1	EMLV/AlHCCR	99	11	3FTx	Mambalgin-3 <i>D. angusticeps</i> ; C0HJB0
				1441.7	1	FCYHNIGMPFR	99	12		

15a	0.1	23.5	1669.8	1	CLPFLFSGCGGNANR	99	7	3FTx	Kunitz-type calciclutidine <i>D. angusticeps</i> ; P81658
15b	1.1	18.3			Negative				
15c.i	0.1	15.0	1797.9 1669.8 1645.8 1010.5 1164.5	1 1 1 1 1	KCLPFLFSGCGGNANR CLPFLFSGCGGNANR WQPPWYCKEPPVR FQTIGEGR WQPPWYCK	99 99 99 95.4 86.5	15 18 14 9 5	BPTI/KUN	Kunitz-type calciclutidine <i>D. angusticeps</i> ; P81658
15c.ii	1.1		2034.9 1288.6	1 1	GCGCPKEMLVAIHCCR EMLVAIHCCR	99 99	7 15	3FTx	Toxin F-VIII <i>D. angusticeps</i> ; P01404
15c.iii			1441.7	1	FCYHNIGMPFR	99	13	3FTx	Mambalgain-3 <i>D. angusticeps</i> ; C0HJB0
16a	trace	21.2	1097.6 1781.9 1413.7	1 1 1	NPNPVPSGCR HWNSTCTTHTFVK CRNPNPVPSGCR	99 99 80.7	6 20 9	NGF	Uncharacter.prot (frag) <i>O.hannah</i> ; V8NP13
16b.i	0.1		996.6 2197.1	1 1	WXYIVPR YSDIWGCATCPKPTNVR	98.6 65.7	9 6	FTx ³	Muscarinic toxin 4 <i>D. angusticeps</i> ; Q9PSN1
	0.5****	18.5	996.6 2197.1	1 1	WXYIVPR YSDIWGCATCPKPTNVR	99 97.7	10 8	3FTx	Muscarinic toxin 4 <i>D. angusticeps</i> ; Q9PSN1
16b.ii			1288.7	1	EMLVAIHCCR	99	9	3FTx	Toxin F-VIII
16b.iii	trace		1669.8	1	CLPFLFSGCGGNANR	99	6	BPTI/KUN	<i>D. angusticeps</i> ; P01404 Kunitz-type calciclutidine <i>D. angusticeps</i> ; P81658
16c.i	1.0	16.2	2358.2 2197.1 2253.0	1 1 1	SIFGITTEDCPDGQNLCFKR SIFGITTEDCPDGQNLCFK GCAATCPIPENYDSIHCK	99 99 99	13 17 21	3FTx	Toxin AdTx1 <i>D. angusticeps</i> ; P85092
16c.ii			2329.2 1124.7	1 1	SIFGITTEPCPDGQNLCFKK KWYIVPR	99 99	14 9	3FTx	Muscarinic toxin 4 <i>D. angusticeps</i> ; Q9PSN1
16c.iii			1291.7	1	NWTFDNIIR	97	8	3FTx	Dendroaspin

			996.6	1	WYVIVPR	93	10		<i>D. janesoni</i> ; P28375
16d	1.4	15.0	2329.2	1	SIFGITTENCPPDQNLCKKK	99	18	3FTx	Muscarinic toxin 4
			1124.7	1	KWYIVPR	99	11		<i>D. angusticeps</i> ; Q9PSN1
			2197.1	1	YSDITWGCAATCPKPTNVR	99	24		
			996.6	1	WYVIVPR	98.6	10		
	trace		1669.8	1	CLPFLFSGCGGNANR	99	11	BPTI/KUN	Kunitz-type calcicludeine
			1645.9	1	WQPPWYCKEYVR	98.5	9		<i>D. angusticeps</i> ; P81658
17a.i	trace	21.5	1097.6	1	NPNPVPSGCR	99	8	NGF	Uncharact.prot (frag)
			1413.7	1	CRNPNPVPSGCR	99	11		<i>O. hannah</i> ; V8NP13
			1709.9	1	FIRIDTACVCVISR	99	8		
			1781.9	1	HWNSYCTTHTFVK	99	15		
17a.ii	0.2		1288.7	1	EMLVAIHCCR	99	9	3FTx	Toxin F-VIII
									<i>D. angusticeps</i> ; P01404
17b	1.4	15.4	2197.1	1	YSDITWGCAATCPKPTNVR	99	16	3FTx	Muscarinic toxin 4
			996.6	1	WYVIVPR	98.9	10		<i>D. angusticeps</i> ; Q9PSN1
18a	2.0	18.0			Negative				
18b	1.4	16.1	2197.1	1	SIFGITTEDCPPDQNLCKFK	99	11	3FTx	Toxin AdTx1
									<i>D. angusticeps</i> ; P85092
19	1.0	15.4	1390.7	1	GCGCPLTLPFLR	99	10	3FTx	Toxin C13S1C1
									<i>D. angusticeps</i> ; P18329
20a.i	0.1	57.4	2053.0	1	TKPAYQFSSCSVQEHQR	99	10	MP	SVMP 1
			1256.7	1	VTL(D ^{tr})LFGKWR	99	14		<i>M. fulvius</i> ; U3EPC7
20a.ii			1297.6	1	SAECPIDSFQR	99	9	MP	SVMP-Hop-13 (Frag)
									<i>H. bungaroides</i> ; R4G7J1
20b	0.1	19.9	1346.6	1	TCEENSCYKR	99	11	3FTx	Toxin C13S1C1
			1319.9	1	SLPKIPLIHGR	99	15		<i>D. angusticeps</i> ; P18329
			1390.7	1	GCGCPLTLPFLR	99	12		
			1333.7	1	GCGPLTLPFLR	96.4	8		
20c	0.5	16.1	1333.7	1	GCGPLTLPFLR	99	11	3FTx	Toxin C13S1C1
			1390.7	1	GCGCPLTLPFLR	99	13		<i>D. angusticeps</i> ; P18329
			1346.6	1	TCEENSCYKR	99	13		

20d	0.7	15.2	1319.9	1	SLPKPLIHGR	99	16	Toxin C13S1C1 <i>D. angusticeps</i> , P18329	
			1346.6	1	TCENSCYKR	99	10		
			1319.9	1	SLPKPLIHGR	99	15		
			1390.7	1	GCGCPLTLPFLR	99	12		
21	2.1	29.9	2551.3	1	YL YVCQYCPAGNIIGSIATPYK	99	14	CRISP <i>Micropechis ikahuku</i> , A0A024AXX20	
			1193.7	1	QIVDKHNALR	99	10		
			1349.8	1	QIVDKHNALRR	62.1	7		
			1537.7	1	NMLQMEWNSDAAQ	99	21		
22.i	1.1	70.7	1234.7	1	VTLNLFGEWR	99	12	MP	SVMP <i>Macrovipera lebetina</i> ; Q98995
22.ii			2053.9	1	TKPAYQFSSCSVQEHQR	99	11	MP	SVMP 1 <i>M. fulvius</i> U3EPC7
22.iii			1508.9	1	RNPQCILNKPRLR	99	11	MP	SVMP
23a.i	0.6	69.5	1234.7	1	VTLNLFGEWR	99	10	MP	SVMP <i>Echis coloratus</i> ; E91G68
23a.ii			1508.8	1	NRPQCILNKPRLR	99	13	MP	SVMP <i>Bothrops erythromelas</i> ; Q8UVG0
23b	0.3	51.3	1256.7	1	VTL(D ^m)LFGKWR	99	13	MP	SVMP 1 <i>M. fulvius</i> U3EPC7
			2053.0	1	TKPAYQFSSCSVQEHQR	99	12		
			1967.0	1	KYIEFYVVVDNKMYYR	99	9	MP	Scuttease-1
24a.i	0.3	74.4	1838.9	1	YIEFYVVVDNKMYYR	96	6		<i>Notechis scutatus</i> ; B5KFFV7
24a.ii			1508.9	1	RNPQCILNKPRLR	99	13	MP	SVMP ussurin <i>Gloydius ussuriensis</i> ; Q7SZD9
24a.iii			2053.1	1	TKPAYQFSSCSVQEHQR	97.7	7	MP	SVMP 1 <i>M. fulvius</i> ; U3EPC7
24b	0.9	50.0	1256.7	1	VTL(D ^m)LFGKWR	99	14	MP	SVMP 1 <i>M. fulvius</i> ; U3EPC7
			2053.0	1	TKPAYQFSSCSVQEHQR	99	14		
25a.i	0.6	71.9	1881.8	1	NGHPCQNNQGYCYNR	99	14	MP	SVMP-Hop-50 (Fragm) <i>Hbungaroides</i> R4G719
25a.ii			1852.9	1	TDIVSPVCGNYFVEVG	99	15	MP	SVMP <i>Ovophis okinavensis</i> ; U3TBS9
25a.iii			1087.6	1	EHQEYLLR	99	10	MP	SVMP <i>atraxe-A.</i>

25b.i	0.2	52.2	1118.6	1	LVLNTFQAGR	99	10	GAL	<i>Naja atra</i> ; D5LMJ3
25b.ii			2053.0 1256.7	1 1	TKPAYQFSSCSVQEHQR VTL(D ^{ms})LFGKWR	99 98.5	9 8	MP	Galectin (Frag) <i>O. hamathi</i> ; V8NHB1 SVMP 1 <i>M. fulvius</i> ; U3EPC7
25c	0.5	22.5			Negative				
26a.i	0.2	70.1	2009.9 1881.8	1 1	NGHPCQNNQGYCYNRK NGHPCQNNQGYCYNR	99 99	9 14	MP	SVMP-Hop-50 (Fragm) <i>H. bangaroidea</i> ; R4G719 SVMP
26a.ii			1852.9	1	TDIVSPVCGNYFEVVG	99	16	MP	<i>Opophis okinawensis</i> ; U3TBSS9
26a.iii			1607.9	1	GATVGLAYVGS LC(N ^{ms})PK	99	15	MP	SVMP-Hem-2 (Frag) <i>Hemiaspis signata</i> ; R4G2W9
26b	0.2	54.4	2412.2 1904.0 2032.0 1243.7	1 1 1 1	TFHGLGVIDWENWRPQWDR HSDSNAFLHLFPESFR KHSDSNAFLHLFPESFR NDQLIWLWR	99 99 99 99	12 12 11 11	HYA	Hyaluronidase <i>M. fulvius</i> ; U3FYZ4
			2780.4	1	APMYPNPEPLVFWNAPTTCQLR	99	17	HYA	Hyaluronidase <i>O. hamathi</i> ; V8P1Z9
26c.i	0.6	39.3	1811.9	1	YIEFYVVVDNEMYK	99	12	MP	SVMP-Sut-51 (Frag) <i>Suta fasciata</i> ; R4FIX4
26c.ii			1234.7	1	VTLNLFGEWR	99	11	MP	SVMP <i>Echis coloratus</i> ; E9JG68
26d.i	0.3	35.9	1759.9	1	VYEMVNALNTMYRR	99	9	MP	SVMP mocarhagin <i>Naja mossambica</i> ; Q10749
26d.ii			1811.9	1	YIEFYVVVDNEMYK	99	12	MP	SVMP-Sut-51 (Frag) <i>Suta fasciata</i> ; R4FIX4
26d.iii			1234.7	1	VTLNLFGEWR	99	9	MP	SVMP <i>Echis coloratus</i> ; E9JG68
26e	0.1	22.8	1118.7 1026.5	1 1	LVLNTFQAGR WGDEQVHK ¹⁰	99 98.3	12 12	GAL	Galectin (Frag) <i>O. hamathi</i> ; V8NHB1
26f	0.1	21.1	1118.6	1	LVLNTFQAGR	99	12	GAL	Galectin (Frag) <i>O. hamathi</i> ; V8NHB1

27a.i	1.0	78.0	1838.9 1967.0	1 1	YIEFYVVVDNKMYYR KYIEFYVVVDNKMYYR	99 99	8 7	MP	Scutellase-1 <i>Notechis scutatus</i> ; B5KFFV7
27a.ii			3059.3	1	(A ^{ox})AKDDCDLPEICTG(Q ^{dh})SAECPMDSFQ _R	99	13	MP	SVMP-Vet17 (Frag) <i>Vermicella annulata</i> ; R4FYV6
27b	0.1	57.2	1462.8	1	TQEL(P ^{ox})SILEFSVGR	99	9	MP	Endoplasmic ret. aminopeptidase 1-like protein <i>Crotalus horridus</i> ; T1E6L9
27c	0.1	45.9	1605.8 1234.7	1 1	SFG(D ^{dh})(W ^{di})RETDLLPR VTNLFGWEWR	99 68.9	12 6	MP	SVMP man SVMP <i>Echis coloratus</i> ; E9JG68
27d	0.1	42.4	1605.8	1	SFG(D ^{dh})(W ^{di})RETDLLPR	99	10	MP	SVMP man
28i	0.5	48.8	1297.6	1	SAECPDTSFQR	99	10	MP	SVMP Australase-1 <i>P. australis</i> ; B5KFFV3
28ii			2191.0	1	(RT ^{dh})KPAYQFSSCSVQEHQR	99	18	MP	SVMP 1 <i>M. fulvius</i> U3EPC7
28iii			2432.1	1	(N ^{dh})LVVAVI(M ^{dh})A(H ^{ox})EMGHNLGIHDDR	99	10	MP	SVMP man
29	0.9	-	-	-	-	-	-	Unknown	-

*Numbers correspond to peaks obtained by RP-HPLC separation; letters (a, b, c, d, e, f) correspond to bands in SDS-PAGE gels; and i, ii, iii correspond to different matching proteins identified in the same electrophoretic band.

** Cysteine residues are carbamidomethylated. Confidence (Conf) and Score (Sc) values are calculated by the Paragon algorithm of ProteinPilot[®], ▼ : reduced SDS-PAGE mass estimations, in kDa. Possible, although unconfirmed/ambiguous amino acid modifications suggested by the automated identification software are shown in parentheses, with the following abbreviations: ^{da}: deamidated; ^{dh}: dehydration; ^{fo}: formylated; ^{ox}: oxidized; ^{na}: Na cation; ^{ca}: carbamidomethyl; ^{di}: deoxidized; Man: manual interpretation of spectrum

*** Protein family abbreviations: 3FTx: three-finger toxin; A₂: CRISP: cysteine-rich secretory protein; HYA: hyaluronidase BPTI/KUN: bovine pancreatic trypsin inhibitor/Kunitz inhibitor; MP: Metalloproteinase; KTC: prokineticin; GAL: galactose binding galectin

***It was not possible to determine the specific percentages of the two proteins of the three finger toxin family using ESI in these bands, as they were of similar mass, however the percentage was attained to the 3FTx in calculating total venom composition.

Table 2: Lethality and Toxicity Score of RP-HPLC fractions of the venom of *D. angusticeps*

Peak	%	Protein family	LD ₅₀ (mg/kg) (95% conf.)	Reported LD ₅₀ (mg/kg)	Toxicity score ¹ (% / LD ₅₀ (kg/mg)
Whole venom	100		0.85 (0.61-1.23)	1.13*	117.6
	70.3	Fractions 4-12 (equal amounts according to mass)	1.36 (0.96-1.66)		51.7
4	2.7	BPTI/Kunitz inhibitor Delta-dendrotoxin <i>D. angusticeps</i> ; P00982 Long epsilon-dendrotox.R55 <i>D. angusticeps</i> ; Q7LZS8	>0.40	Delta- dendrotoxin: 15 [43]	<7
5	4.8	BPTI/Kunitz inhibitor Alpha-dendrotoxin <i>D. angusticeps</i> ; P00980	>0.71	23 [44]	<7
6	³ 0 (1:1 mix)	BPTI/Kunitz inhibitor Alpha-dendrotoxin <i>D. angusticeps</i> ; P00980 3FTx Muscarinic toxin 2 <i>D. angusticeps</i> ; P18328	>0.45		<7
7	6.2	3FTx Thrombostatin <i>D. angusticeps</i> ; P81946	>0.92		<7
8	6.3	3FTx Fasciculin-2 <i>D. angusticeps</i> ; P0C1Z0 Thrombostatin <i>D. angusticeps</i> ; P81946 Rho-elapitoxin-Dalb <i>D. angusticeps</i> ; P86419	0.58 (0.17-1.23)	Fasciculin 2 >20 [33]	10.9
9	¹⁵ 9 (2:1:2 mix)	BPTI/Kunitz inhibitor Protease inhibitor 1 <i>W. aegyptia</i> ; C1IC50	>2.38		<7

		3FTx Fasciculin-1 <i>D.angusticeps</i> ; P0C1Y9 Synergistic-like protein <i>D.angusticeps</i> ; P17696		
10	6.8	3FTx Fasciculin-1 <i>D.angusticeps</i> ; P0C1Y9 Synergistic-like protein <i>D.angusticeps</i> ; P17696 Thrombosstatin <i>D.angusticeps</i> ; P81946 Dendotoxin A (fragm) <i>D.angusticeps</i> ; Q9P508	>2.11	<3.3
11	16.8	3FTx Toxin F-VIII <i>D.angusticeps</i> ; P01404	>2.52	<7
12	^{7.7} (2:2:1 mix)	3FTx Mambalgin-3 <i>D.angusticeps</i> ; C0HJB0 Toxin F-VIII <i>D.angusticeps</i> ; P01404 BPTI/Kunitz inhibitor Kunitz-type calcicludeine <i>D.angusticeps</i> ; P81658	>1.1	<7
13	2.1	3FTx Mambalgin-3 <i>D.angusticeps</i> ; C0HJB0 Toxin F-VIII <i>D.angusticeps</i> ; P01404	>0.64	<7
14	1.7	3FTx Mambalgin-3 <i>D.angusticeps</i> ; C0HJB0 Toxin F-VIII <i>D.angusticeps</i> ; P01404 BPTI/Kunitz inhibitor Kunitz-type calcicludeine <i>D.angusticeps</i> ; P81658	>0.26	<7
15	2.4	3FTx Mambalgin-3 <i>D.angusticeps</i> ; C0HJB0 Toxin F-VIII <i>D.angusticeps</i> ; P01404	>0.35	<7

16	3.0	3FTx Toxin F-VIII <i>D. angusticeps</i> ; P01404 Toxin AdTx1 <i>D. angusticeps</i> ; P85092 Muscarinic toxin 4 <i>D. angusticeps</i> ; Q9PSN1	>0.44	<7
17	1.6	3FTx Toxin F-VIII <i>D. angusticeps</i> ; P01404 Muscarinic toxin 4 <i>D. angusticeps</i> ; Q9PSN1	>0.24	<7
18	3.4	3FTx Toxin AdTx1 <i>D. angusticeps</i> ; P85092	>0.50	<7
19	1.0	3FTx Toxin C13S1C1 <i>D. angusticeps</i> ; P18329	>0.15	<7
20	1.4	3FTx Toxin C13S1C1 <i>D. angusticeps</i> ; P18329	>0.21	<7
21	2.1	CRiSP CRiSP <i>Micropechis ikaheka</i> ; A0A024AX20	>0.31	<7

*. <http://snakedatabase.org/pages/LD50.php#legendAndDefinitions>

¹Toxicity Score was defined as the ratio of protein fraction abundance (%) in the venom divided by its estimated median lethal dose (LD₅₀) for CD-1 mice by i.v. injection. In the case of crude venom, the % abundance was 100%.

²Mix indicates that the fraction did not contain a pure, isolated toxin, but instead a mixture of 2–4 different toxins in variable ratios indicated in the table.

Figure 1

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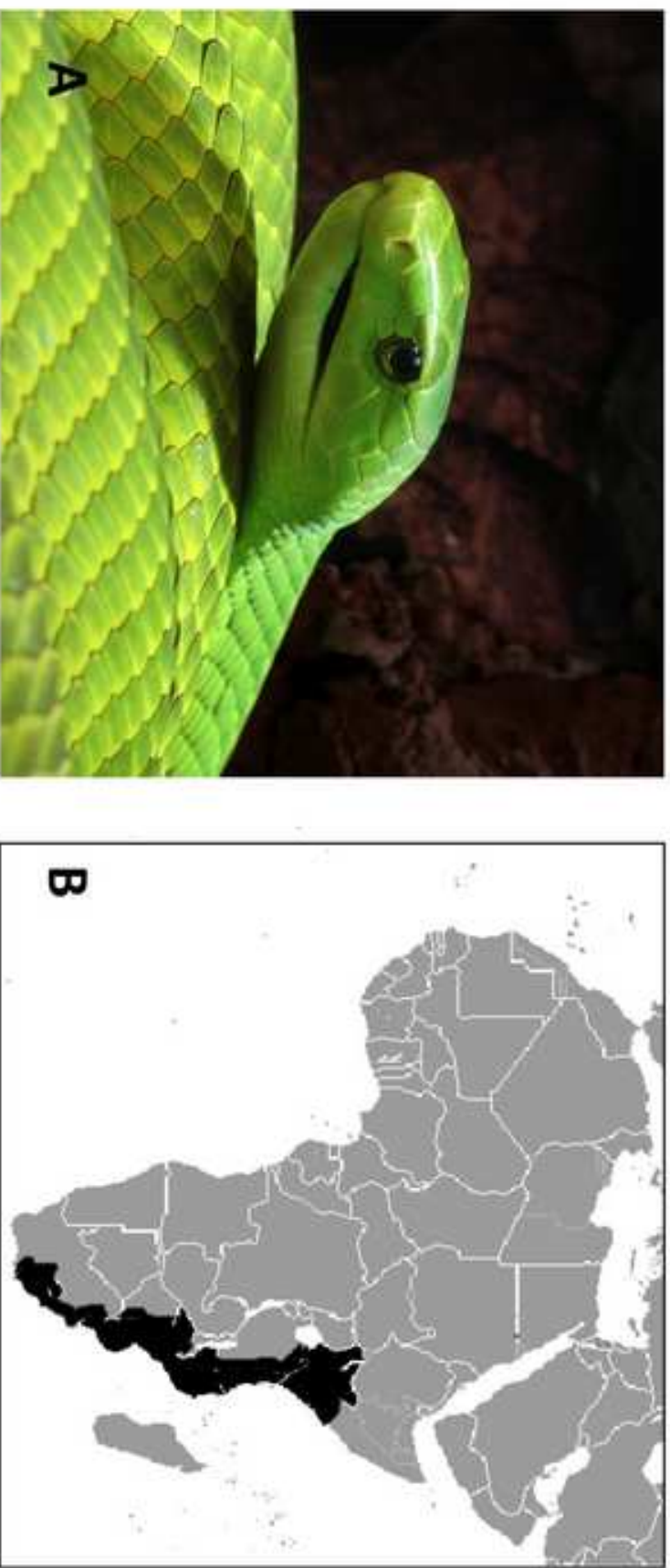


Figure 2

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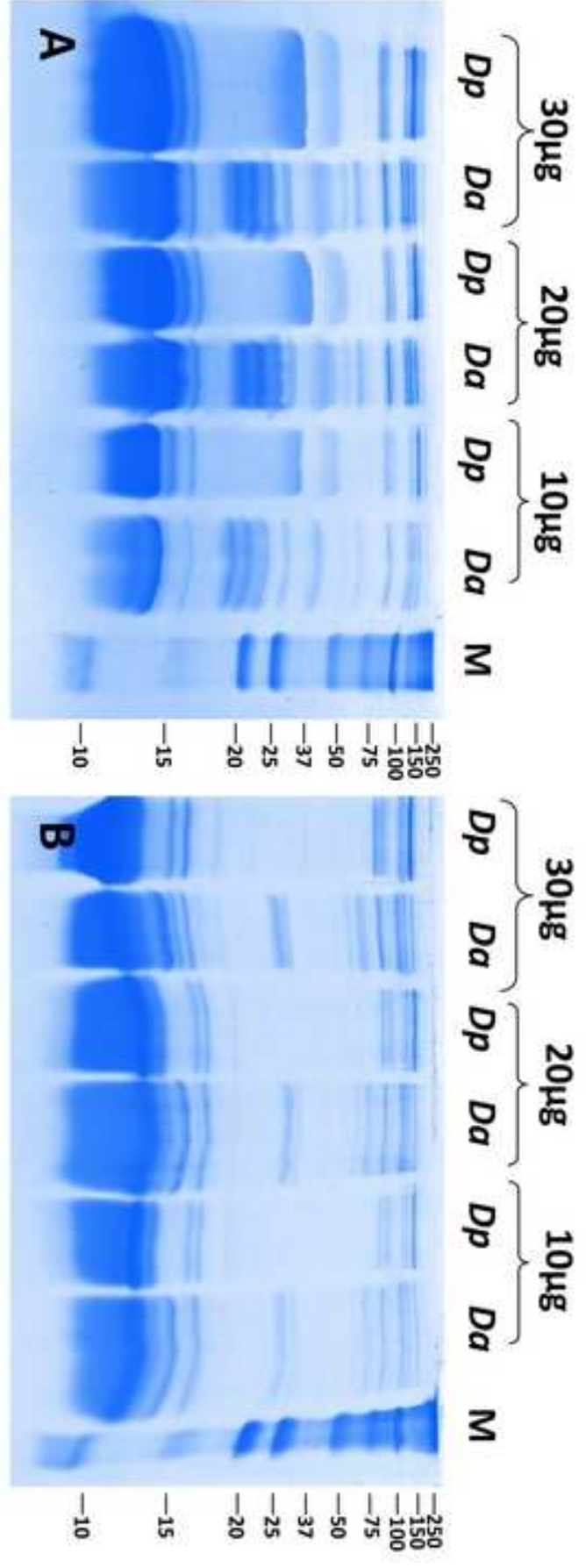


Figure 3
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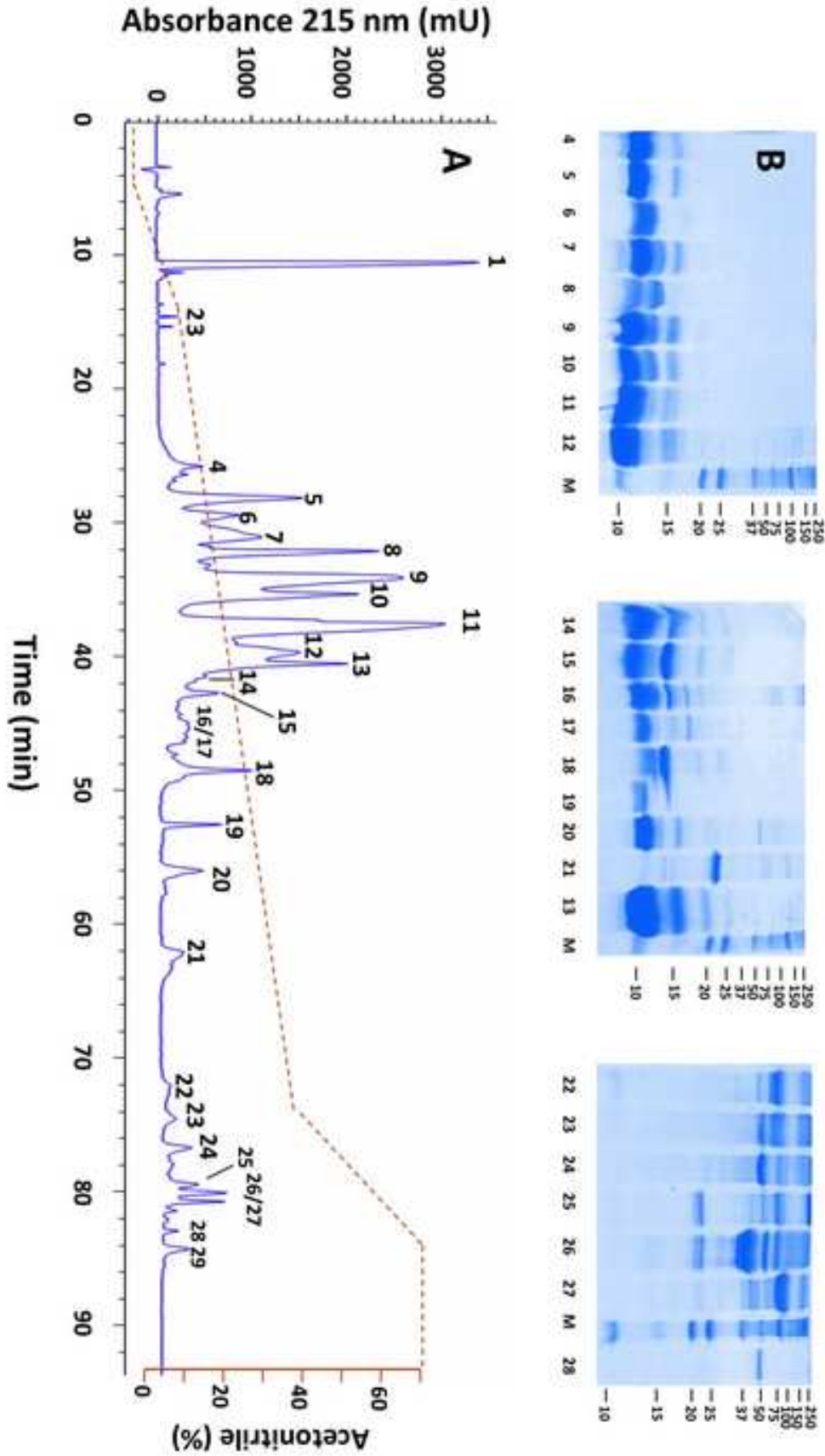


Figure 4
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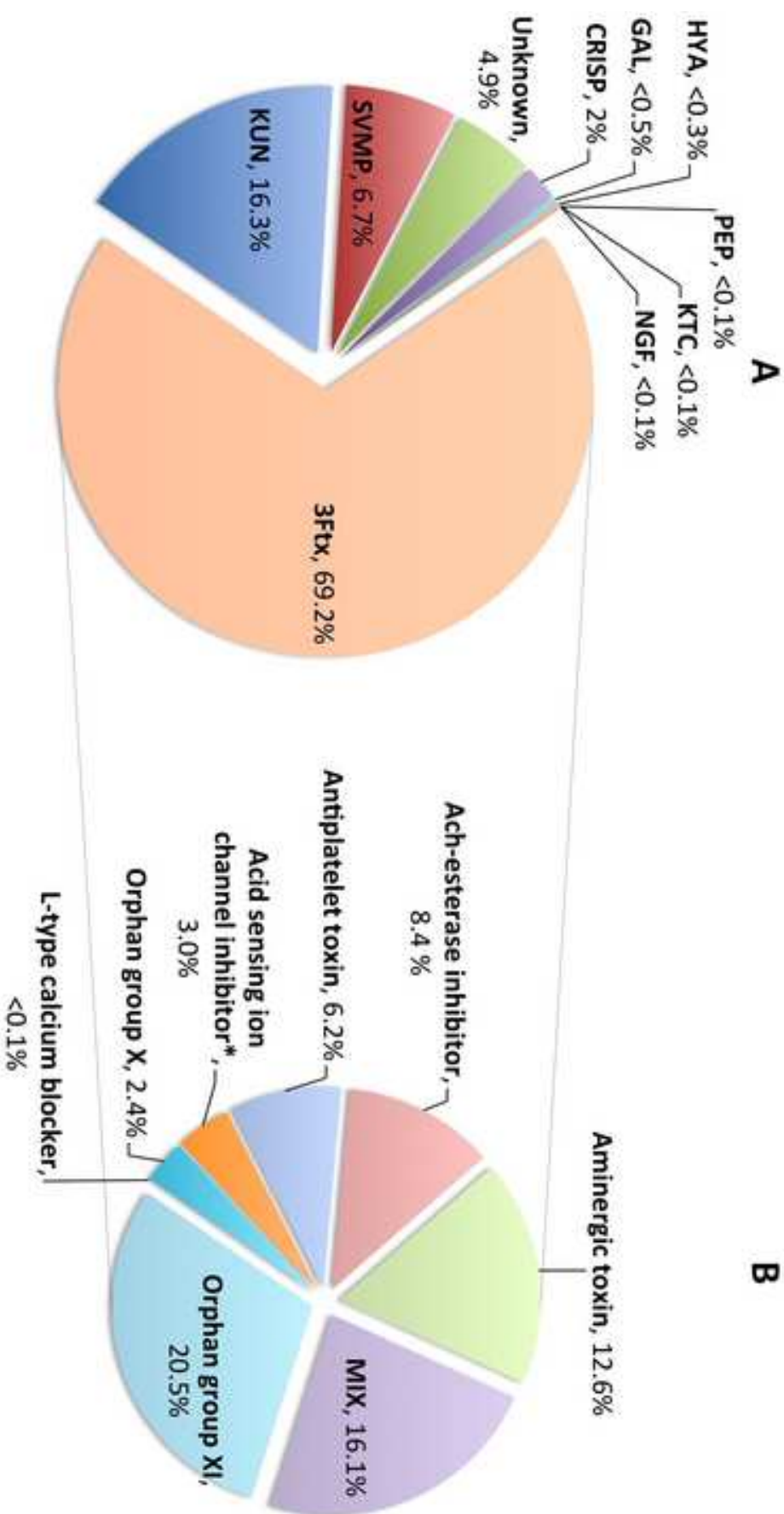


Figure 5
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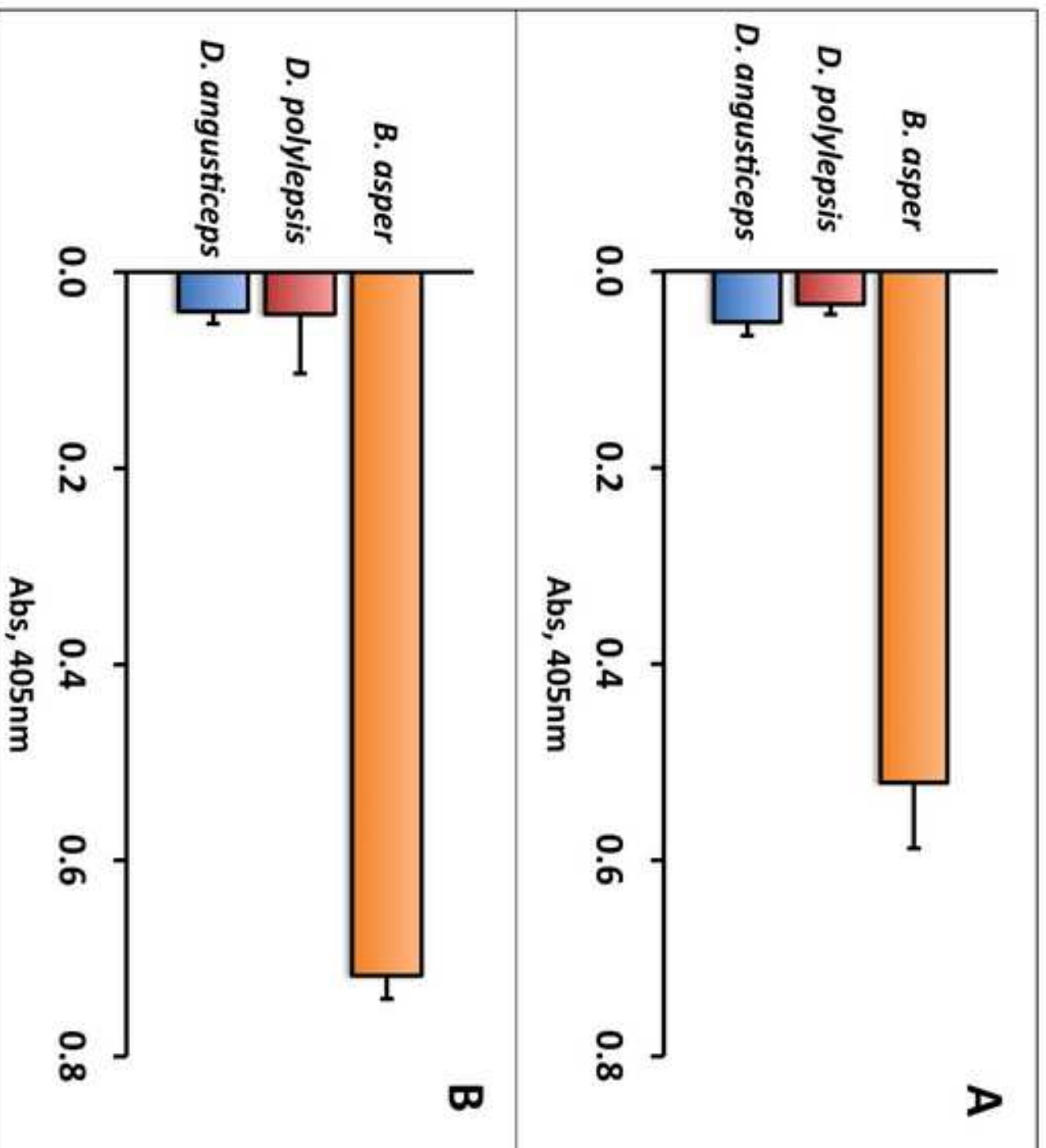


Figure 6
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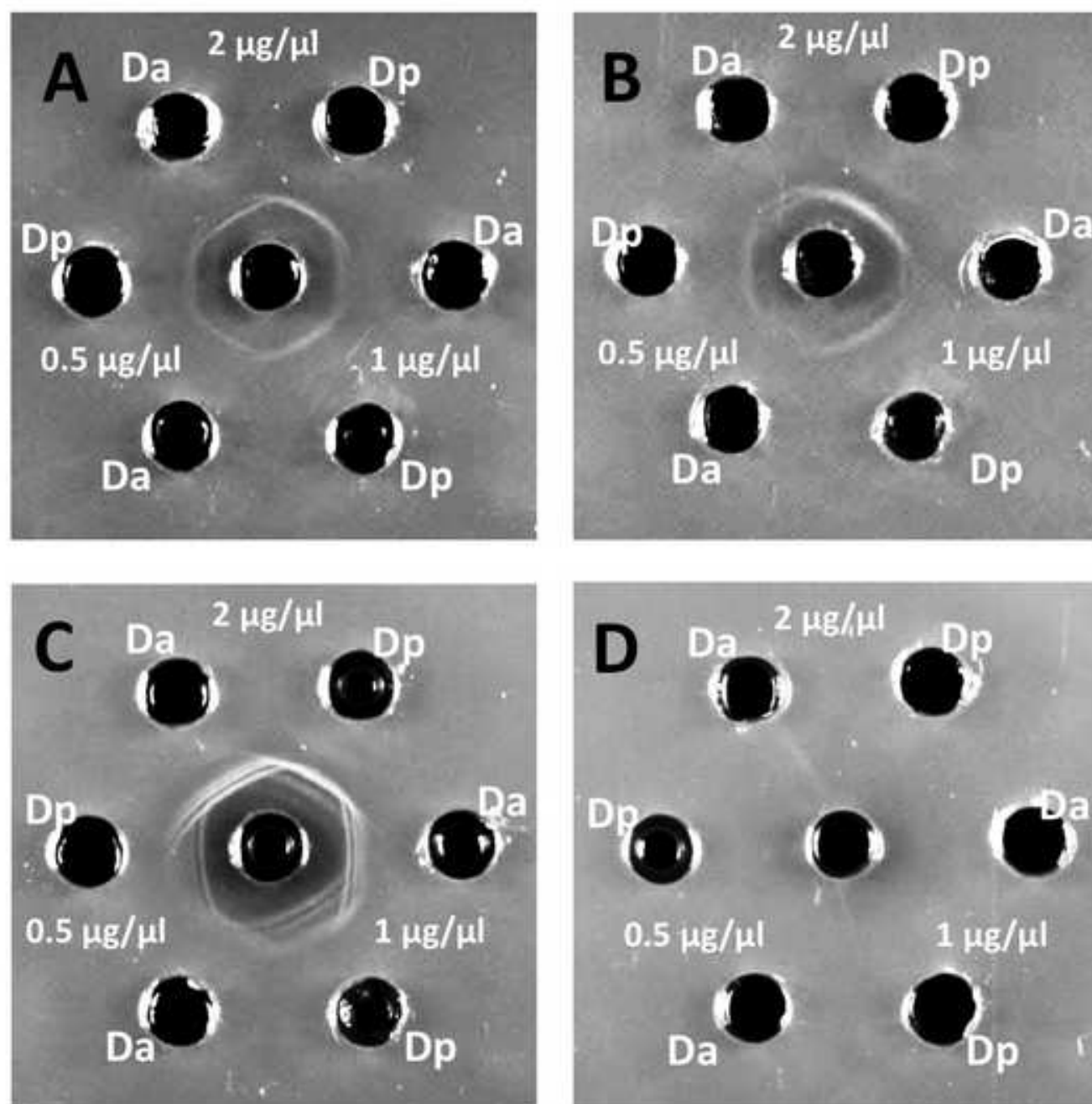


Figure 7
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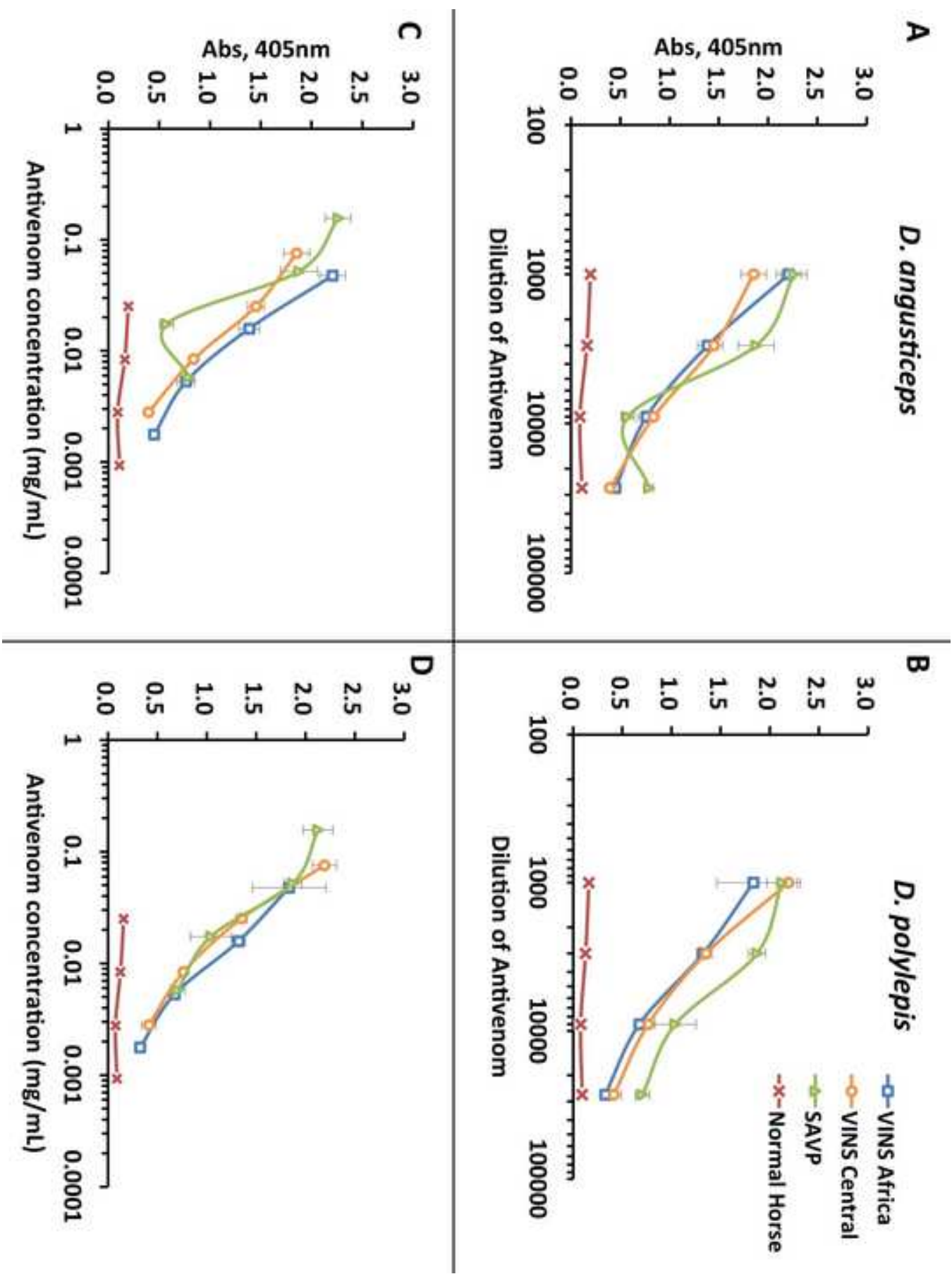
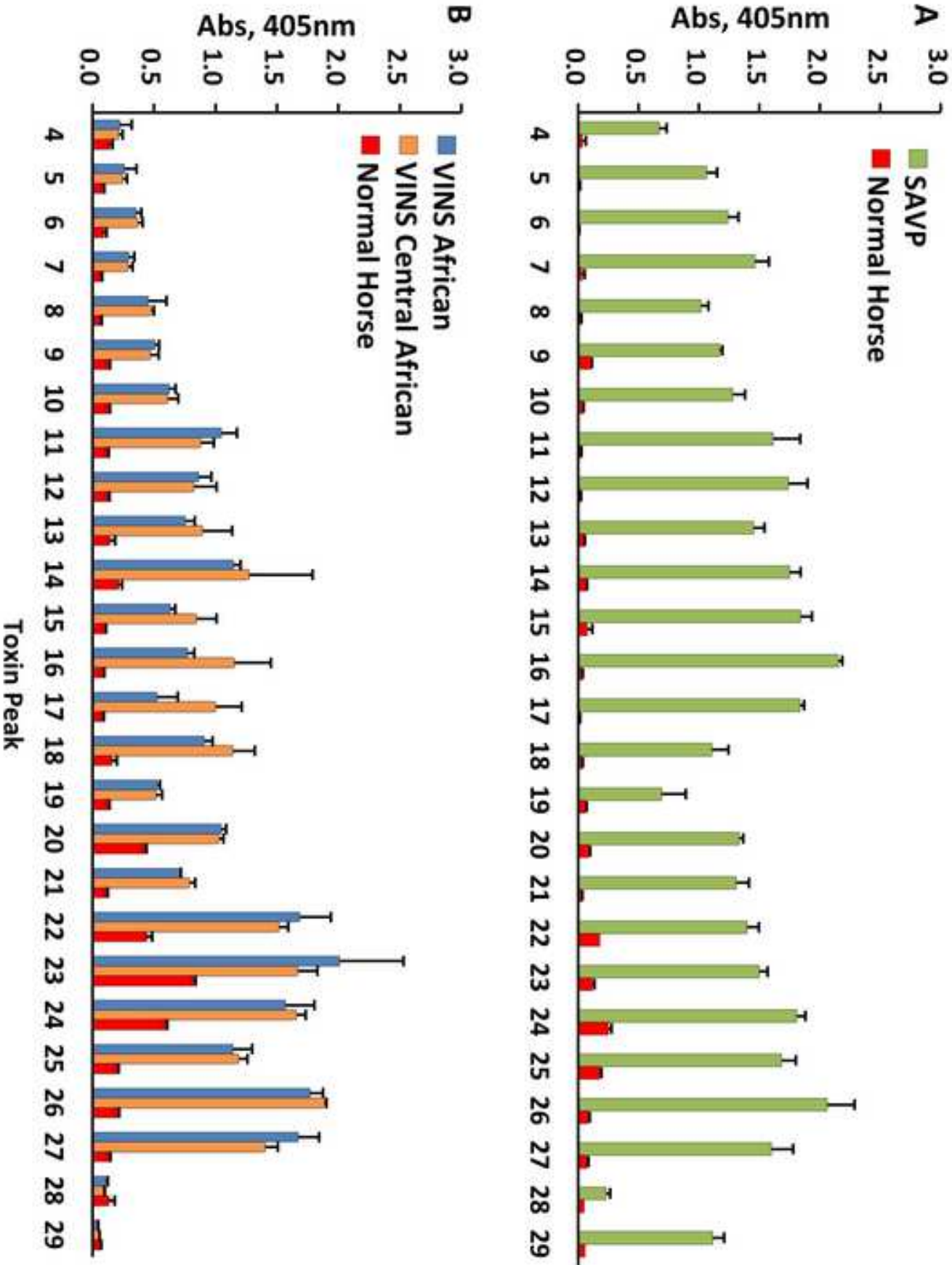


Figure 8
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